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Selinene volatiles are essential precursors for maize defense promoting fungal pathogen resistance

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One Sentence Summary: Maize terpene synthase 21 encodes a β -selinene synthase enabling the production of antifungal defenses.

List of Author contributions:

E.A.S., A.H. and Y.D. conceived the original screening and research plans; Y.D. performed most of the experiments; P.W. provided technical assistance to Y.D.; Y.D., T.G.K., C.A.M.R., J.L.S., A.E.L. designed the experiments and analyzed the data; E.A.S, Y.D. and A.H. conceived the project and wrote the article with contributions of all the authors; T.G.K., C.A.M.R., A.E.L. supervised and complemented the writing of specific sections.

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46 **Abstract**

To ensure food security, maize (*Zea mays*) is a model crop for understanding useful traits underlying stress resistance. In contrast to foliar biochemicals, root defenses limiting the spread of disease remain poorly described. To better understand below-ground defenses in the field, we performed root metabolomic profiling and uncovered unexpectedly high levels of the sesquiterpene volatile β -selinene and the corresponding non-volatile antibiotic derivative, β -costic acid. The application of metabolite-based Quantitative Trait Loci (mQTL) mapping using bi-parental populations, genome wide association studies, and near-isogenic lines (NILs) enabled the identification of terpene synthase 21 (*ZmTps21*) on chromosome 9 as a β -costic acid pathway candidate gene. Numerous closely examined β -costic acid deficient inbred lines were found to harbor *Zmtps21* pseudo genes lacking conserved motifs required for farnesyl diphosphate (FPP) cyclase activity. For biochemical validation, a full length *ZmTps21* was cloned, heterologously expressed in *E. coli* and demonstrated to cyclize FPP yielding β -selinene as the dominant product. Consistent with microbial defense pathways, *ZmTps21* transcripts strongly accumulate following fungal elicitation. Challenged field roots containing functional *ZmTps21* alleles displayed β -costic acid levels over 100 $\mu\text{g g}^{-1}$ FW, greatly exceeding *in vitro* concentrations required to inhibit the growth of five different fungal pathogens and rootworm larvae (*Diabrotica balteata*). *In vivo* disease resistance assays, using *ZmTps21* and *Zmtps21* NILs, further support the endogenous antifungal role of selinene-derived metabolites. Involved in the biosynthesis of non-volatile antibiotics, *ZmTps21* exists as a useful gene for germplasm improvement programs targeting optimized biotic stress resistance.

Introduction

Plants are protected from a broad range of harmful biotic agents by initial perception events, signal transduction cascades and the elicitation of defense metabolism (Vanetten et al., 1994; Harborne, 1999; Dangl et al., 2013; Huffaker et al., 2013). In maize (*Zea mays*), seedlings are largely protected from attack by a complex suite of hydroxamic acid-based defenses, termed benzoxazinoids (BX), responsible for resistance to diverse threats spanning fungal pathogens and herbivores including Northern corn leaf blight (*Setosphaeria turctica*) and the European corn borer (ECB; *Ostrinia nubilalis*) (Beck et al., 1957; Couture et al., 1971; McMullen et al., 2009). Sixty years of research has resulted in a nearly complete metabolic and genetic BX pathway in maize involving over a dozen individual enzymes and metabolites (Frey et al., 2009; Meihls et al., 2013; Handrick et al., 2016). Additionally, diverse terpenoids and underlying terpene synthases (Tps) have also been demonstrated to play important protective roles (Degenhardt, 2009; Schmelz et al., 2014). As indirect defenses, herbivore-elicited terpene volatiles can function as diffusible signals to attract natural enemies, such as parasitoids and entomopathogenic nematodes, to above- and below-ground insect pests, respectively (Rasmann et al., 2005; Schnee et al., 2006).

Of the many biosynthetic classes of natural products, terpenoids are the most structurally diverse with well over 25,000 established compounds. In addition to roles as phytohormone signals, specialized terpenoids mediate inter-organism interactions and serve as chemical barriers (Gershenzon and Dudareva, 2007). In maize, terpene olefins are nearly ubiquitous components of

98 induced above and below-ground volatile emissions acting as indirect plant
99 defenses following biotic stress (Turlings et al., 1990; Degenhardt, 2009;
100 Degenhardt et al., 2009; Kollner et al., 2013). Maize terpene olefins can also
101 serve as precursors for localized production of non-volatile antibiotic terpenoid
102 defenses (Schmelz et al., 2014). While often undetectable at the level of volatile
103 pathway intermediates, the inducible accumulation of non-volatile terpenoid end-
104 products can limit the damage caused by fungi, herbivores and oxidative
105 stresses (Harborne, 1999; Ahuja et al., 2012). Despite significant advances,
106 continuing discoveries in maize reveals that our collective knowledge of
107 biochemical defenses and pathway genes responsible for mitigating crop stress
108 remains incomplete.

109 Decades of intensive research in related Poaceous crops, such as rice
110 (*Oryza sativa*), has revealed multiple pathways of inducible labdane-related
111 diterpenoids including momilactones, oryzalexins, and phytocassanes that
112 underlay protective responses to biotic and abiotic stress (Schmelz et al., 2014).
113 More recently, complex arrays of acidic terpenoids have been detected in maize
114 and include sesquiterpenoids derived from β -macrocarpene and diterpenoids
115 derived from *ent*-kauranes, termed zealexins and kauralexins, respectively
116 (Huffaker et al., 2011; Schmelz et al., 2014). From a biosynthetic and pathway
117 perspective, maize genes underlying the production of antifungal agents remain
118 largely unknown. In the case of maize diterpenoid defenses, a specific *ent*-
119 copalyl diphosphate synthase (Anther ear 2; ZmAn2), is the only enzyme

demonstrated *in planta* essential for kauralexin biosynthesis (Vaughan et al., 2015).

To uncover further defense pathways, we employed targeted metabolomic profiling on field grown maize roots naturally exposed to combinations of herbivores and pathogens (Baldwin, 2012). Curiously, high levels of rarely encountered eudesmane sesquiterpenoids including β -selinene and β -costic acid dominated the chemical profiles of many samples. While not previously associated with maize, β -costic acid is known from the Asteraceae family, including false yellowhead (*Dittrichia viscosa*) and costus (*Saussurea costus*), and has been utilized in extracts for potent antibiotic activities against diverse organisms (Rao and Alvarez, 1981; Wu et al., 2006; Katerinopoulos et al., 2011). Despite the diverse phylogenetic occurrence in nature, a specific pathway predominantly leading to β -costic acid has not been described in plants. To explore the maize β -costic acid pathway, combined genetic mapping approaches with the intermated B73 x Mo17 (IBM) population of recombinant inbred lines (RILs) (Lee et al., 2002), the Goodman diversity panel (Flint-Garcia et al., 2005), and IBM near-isogenic lines (NILs) (Eichten et al., 2011) were used for metabolite-based Quantitative Trait Loci (mQTL) mapping. Biochemical characterization of the mQTL-identified Tps candidate utilized heterologous expression in *E. coli* to confirm identification of a comparatively product-specific β -selinene synthase. Transcript expression and metabolite analyses following elicitation with multiple pathogens and western corn rootworm (WCR, *Diabrotica virgifera virgifera*) larvae (Gray et al., 2009; Meinke et al., 2009; Miller et al.,

2009; Spencer et al., 2009; Tinsley et al., 2013) were used to assess pathway activation. Concentrations of β -costic acid below those detected in field tissues were then used to examine *in vitro* antibiotic activity against 5 fungal species. Similarly, NILs were used to investigate *in vivo* root resistance following challenge with *Fusarium verticillioides* and *Fusarium graminearum*. Collectively our results support the existence of a previously unrecognized β -costic acid pathway in maize that contributes to fungal pathogen resistance.

RESULTS

Identification of α and β -selinene derived products as inducible maize sesquiterpenoids that can influence generalist root herbivores. Our previous investigation of maize responses following stem herbivory and fungal elicitation enabled the discovery of two distinct biosynthetic classes of inducible acidic terpenoids (Huffaker et al., 2011; Schmelz et al., 2011). Similarly, experiments examining maize root defenses elicited by banded cucumber beetle (*Diabrotica balteata*) larvae and *F. verticillioides* infection confirmed shared responses in diverse tissue types (Vaughan et al., 2015). Given that predominant defenses change over ontogeny and that controlled lab experiments do not capture the full suite of biotic stresses in nature (Kollner et al., 2004; Baldwin, 2012), we sought to expand our targeted metabolomic analyses to roots in the context of natural biotic challenge (Schmelz et al., 2004). As expected, mature visibly-necrotic roots of field-challenged maize lines including hybrid sweet corn (var. Golden Queen) and the inbred Mo17 contained zealexins (Fig. 1A); however, chemically

166 extracted samples unexpectedly also contained α -selinene, β -selinene, β -costol,
167 α -costic acid and β -costic acid (Fig. 1; Supplemental Fig. S1). In volatile
168 collections of live Mo17 root emissions, α -selinene, β -selinene (Fig. 2) and the
169 aldehyde β -costal (Supplemental Fig. S1) were likewise detectable. As the major
170 analyte, live field-collected Mo17 roots displaying visible necrosis emit
171 predominantly β -selinene (Fig. 2). In contrast, β -selinene emission is absent in
172 B73 roots; however, production reappears in select B73 x Mo17 RILs, for
173 example IBM0287 (Fig. 2). Similar volatile emission results are observed in live
174 Mo17 stems following inoculation with the necrotrophic fungal pathogen
175 (*Cochliobolus heterostrophus*), commonly known as southern leaf blight (SLB)
176 (Fig. 2). Consistent with root metabolite patterns, the reference genome inbred
177 B73 (Schnable, 2009) remains void of α and β -selinene stem volatiles under
178 identical conditions (Fig. 2). Qualitative metabolite differences between B73,
179 Mo17 and select RILs provides empirical evidence for genetic variation in
180 selinene biosynthesis and encourages the use of genetic mapping resources
181 (Lee et al., 2002).

182 Our quantification of unexpectedly high levels of β -selinene and β -costic
183 acid in field collected maize roots was paired with casual field observations of
184 adult *D. balteata* beetles on leaves. Given the broad host range of *D. balteata*
185 larvae (Saba, 1970) and pest pressures exerted by western corn rootworm
186 (WCR: *Diabrotica virgifera virgifera*) larvae including the promotion of secondary
187 disease (Flint-Garcia et al., 2009; Gray et al., 2009), we conducted controlled
188 *Diabrotica-maize* interaction experiments. In growth chamber assays, tissue

extracts of roots revealed both β -selinene and β -costic acid following damage by WCR larvae (Fig. 2). Given the high levels of selinene-derived metabolites observed in field-collected roots, additional assessments of WCR and *D. balteata* preference and performance were conducted on larvae. For both *Diabrotica* species, we observed no influence of exogenously applied β -costic acid on root preference but found a significant inhibitory role of β -costic acid on *D. balteata* performance (Fig. 2).

Combined linkage and association mapping identifies maize terpene synthase *ZmTps21* as a candidate biosynthetic gene. β -selinene has been previously detected in the volatile profiles of pathogen-challenged maize tissue; however, the biosynthetic source and physiological function(s) have not been elucidated (Becker et al., 2014). Given our observation that selinene-derived pathway products can predominate in maize under specific conditions, we sought to identify the gene(s) responsible. We first employed the IBM-RILs for mQTL mapping. As a predictable non-volatile pathway end product, β -costic acid levels were analyzed in naturally challenged roots of 216 IBM RILs (Supplemental Table S1). Composite Interval Mapping (CIM) placed the locus in Bin 9.05 (Fig. 3) (Gardiner et al., 1993). For comparative purposes, the IBM-RIL data was also explored using 173,984 SNPs and association mapping via a General Linear Model (GLM) (Bradbury et al., 2007) and Unified Mixed Linear Model (MLM) (Yu et al., 2006). All approaches supported a single statistically significant locus on chromosome 9 (Fig. 3, Supplemental Fig. S2). Additionally we performed an

elicited metabolite-based Genome-Wide Association Study (mGWAS) using β -costic acid levels in greenhouse grown inbreds from the Goodman diversity panel (Flint-Garcia et al., 2005). Similarly, we detected a single statistically significant locus on chromosome 9 (Fig. 3). An independent mGWAS replication conducted with field grown plants yielded an identical result (Supplemental Fig. S2). Correspondence of physical QTL coordinates identified with IBM RILs and the replicated GWAS results (Fig. 3, Supplemental Fig. S2) robustly supported a single narrow locus controlling maize β -costic acid levels.

For additional confirmation, select B73xMo17 near isogenic lines (NILs) were analyzed following stem elicitation (Eichten et al., 2011). B73 chromosomal segments introgressed into Mo17 dominating lines (specifically m012, m048, m050 and m062) were each deficient in the production of β -costic acid (Fig. 3). In contrast, β -costic acid production in NILs with introgressions of the Mo17 allele into the B73 genetic background (lines b047, b055, b069, and b157) were similar to Mo17 (Fig. 3, Supplemental Fig. S3), confirming existence of the β -costic acid associated locus in Bin 9.05. Further analyses of additional NIL lines (b022, b033, m002, m065, and m092) narrowed the locus to 13 predicted genes isolated on bacterial artificial chromosome (BAC) clones, AC213878 and AC204415 (Fig. 3). Of the remaining candidates, only a single uncharacterized gene (GRMZM2G011151) displayed significant sequence homology with known terpene synthases. Supportively, detailed examination of 3 independent association mapping results likewise demonstrated the presence of highly significant SNPs associated with the Tps candidate (GRMZM2G011151), termed

ZmTps21 (Supplemental Fig. S2). Genomic structure analysis of B73 *Zmtps21* revealed three predicted exons encoding a 297 amino acid protein lacking the conserved Tps catalytic domains, namely the DDXXD and RXR motifs (Fig. 3), which are essential for function (Chen et al., 2011). Collectively, these findings made B73 *Zmtps21* a parsimonious inactive β -selinene synthase pseudogene candidate meriting further examination.

In an attempt to isolate the Mo17 *ZmTps21* cDNA sequence, early RT-PCR trials with primers based upon B73 *Zmtps21* cDNA failed due to nucleotide polymorphisms. Eventually a segment near the Mo17 *ZmTps21* 5'-end of genomic DNA was obtained by a PCR and sequenced. Alignments revealed that the segment near the Mo17 *ZmTps21* 5'-end DNA fragment shared high sequence similarity to that of B73 *Zmtps21*. Therefore, the 5'-end cDNA sequence of Mo17 *ZmTps21* was obtained by RT-PCR and extended by PCR with rapid amplification of cDNA ends (rPCR) using a cDNA library to obtain the full-length Mo17 *ZmTps21* cDNA (Fig. 3; Supplemental Fig. S4). The deduced amino acid sequence of the open reading frame contained the conserved terpene synthase domains including the DDXXD (residues 325 to 329) and RXR (residues 288 to 290) motifs (Supplemental Fig. S4). The amino acid sequence of *ZmTps21* resembles (<60% identity) those of other plant sesquiterpene synthases and shares less than 40% sequence identity with previously characterized maize sesquiterpene synthases, such as *ZmTps6*, *ZmTps10*, *ZmTps11*, and *ZmTps23* (Supplemental Fig. S4). With only 30% identity at the amino acid level, Mo17 *ZmTps21* is even more distantly related to the *Ocimum*

basilicum sesquiterpene synthase (SES) which produces detectable levels of β -selinene as part of a complex blend (Supplemental Fig. S4) (Iijima et al., 2004).

To understand the extent of genetic variation in *ZmTps21* alleles, we examined 15 commonly investigated inbreds. *ZmTps21* genomic sequences were isolated by PCR using primers based on the B73-*Zmtps21* and Mo17-*ZmTps21* genome sequences, respectively (Supplemental Table S1). Sequence analyses demonstrated that the *Zmtps21* alleles from B73-like lines (Ki3, M37W, MS71, M162W, CML247, Ki11, Mo18W) share >98% DNA sequence identity and basic genome structure, whereas Mo17-like *ZmTps21* alleles (Hp301, TX303, Oh43, Oh7B, Ky21 and W22) contain six exons and share higher than 98% sequence identity at the amino acid level (Supplemental Fig. S5 and S6). These results support the hypothesis that B73-like inbred lines share a common mutation ancestry.

***In vitro* assays demonstrate that ZmTps21 is a largely product-specific β -selinene synthase.** *ZmTps21* lacks a predicted N-terminal transit peptide suggesting that the enzyme is not targeted to plastids as is typical of monoterpene and diterpene synthases, but instead remains cytosolic consistent with predictions for a sesquiterpene synthase (Gershenzon, 1999). To obtain additional support for the hypothesis that Mo17 *ZmTps21* is a β -selinene synthase, heterologous expression was performed in *E. coli* and the resulting protein extract was incubated with the precursor substrate (*E,E*-farnesyl diphosphate (FPP)). β -selinene is the dominant product observed by GC-MS

281 along with several other minor sesquiterpene olefins, including α -selinene and β -
282 elemene (Fig. 4). Thus *ZmTps21* encodes a selinene synthase with predominant
283 β -selinene product specificity that includes α -selinene as a minor product
284 consistent with the olefin and oxygenated metabolite ratios observed *in planta*
285 (Fig. 1, Supplemental Fig. S1). Injection of the *ZmTps21* reaction products on a
286 GC column at different temperatures revealed that the β -elemene present is due
287 to a Cope rearrangement of germacrene A (Supplemental Fig. S7 and S8) (de
288 Kraker et al., 2001). Germacrene A also is a neutral reaction intermediate of the
289 tobacco (*Nicotiana tabacum*) enzyme 5-*epi*-aristolochene synthase (TEAS)
290 responsible for the pathogen-elicited biosynthesis of capsidiol (Cane, 1990;
291 Starks et al., 1997). The enzymatic protonation of germacrene A leads to the
292 eudesmane carbocation further converted to 5-*epi*-aristolochene. Given that β -
293 selinene is simply formed by a deprotonation of a eudesmane carbocation, it
294 likely that the reaction catalyzed by *ZmTps21* also includes the formation and
295 protonation of germacrene A. A sequence comparison of *ZmTps21* with TEAS
296 and other Tps able to protonate neutral reaction intermediates demonstrate that
297 the amino acids of the catalytic triad involved in the protonation reaction are
298 conserved (Starks et al., 1997) (Supplemental Fig. S4). Curiously, two *ZmTps21*
299 mutants with altered C-termini obtained as cloning artifacts produced only
300 germacrene A (Supplemental Fig. S7 and S8), suggesting additional influence of
301 the C-terminus on the protonation reaction and specificity of the final product.

***ZmTps21* transcripts are pathogen inducible and correspond with β -costic acid accumulation.** To examine endogenous patterns, we compared Mo17 *ZmTps21* expression with established *ZmTps6/11* expression associated with zealexin biosynthesis (Kollner et al., 2008; Huffaker et al., 2011). Similar to *ZmTps6/11*-zealexin relationships, *ZmTps21* transcripts and β -costic acid are barely detectable in control tissues and not significantly elicited by mechanical damage alone (Fig. 5). After elicitation with heat-killed *Fusarium* hyphae, *ZmTps6/11* transcripts reached maximal levels at day 1 while *ZmTps21* transcripts levels continued to accumulate for an additional day (Fig. 5). Zealexin A1 was readily detectable at day 1 and continued to increase over the 4 days while β -costic acid accumulation was first detected at day 2 reaching similar levels at day 4 (Fig. 5). Thus, *ZmTps21* transcripts and product accumulation display longer-term and temporally layered elicitation kinetics alongside the zealexin biosynthetic pathway.

To examine whether *ZmTps21* transcripts and β -costic acid levels change specifically in response to aggressive pathogens such as *C. heterostrophus* and *F. verticillioides* or whether the response also follows opportunistic fungi such as *Rhizopus microsporus* and *Aspergillus parasiticus*, both parameters were analyzed in inoculated stems. Exposure to *C. heterostrophus*, *F. verticillioides*, *R. microsporus*, and *A. parasiticus* all resulted in significant induction of both *ZmTps6/11* transcript levels and zealexin A1, which vary in response to different fungi (Fig. 5) (Huffaker et al., 2011). In the same context the 4 fungal species also led to significant accumulation of *ZmTps21* transcripts and β -costic acid

(Fig. 5). Infection with *C. heterostrophus* led to the highest induction of both *ZmTps21* transcripts and β -costic acid in stems, similar to *ZmTps6/11* transcripts and zealexin levels, respectively (Fig. 5). To further consider the natural occurrence of β -selinene derived metabolites in diverse inbreds (McMullen et al., 2009) we analyzed the scutella tissues of 10-d old seedling plants. β -costic acid was detected in all lines harboring Mo17-like *ZmTps21* alleles (Hp301, TX303, Oh43, Oh7B, Ky21, and W22) and was comparatively absent from all inbreds harboring B73-like *Zmtps21* (Ki3, M37W, MS71, M162W, CML247, Ki11, Mo18W) pseudogenes (Fig. 5; Supplemental Fig. S5 and S6). Collectively, these results support the existence of a single β -selinene synthase in maize responsible for the production of β -costic acid.

***In vitro* and *in vivo* assays support a defensive role for β -costic acid in fungal disease resistance.** In effort to assess physiological roles, we quantified β -costic acid present in replications of field-collected roots of B73, Sweet corn (var. Golden Queen), Mo17 and the *ZmTps21* IBM-RIL 0287. On average, sectors of roots containing visible necrosis from each responsive line contained well over 100 $\mu\text{g g}^{-1}$ FW of β -costic acid (Fig. 5). Using this conservative baseline, we then examined the antimicrobial activity of β -costic acid against *F. verticillioides*, *F. graminearum*, *R. microsporus*, *A. parasiticus*, and *C. heterostrophus* in liquid culture assays. At 100 $\mu\text{g ml}^{-1}$, β -costic acid completely inhibited the growth of *R. microsporus* and significantly suppressed the growth of all other fungi (Fig. 6; Supplemental Fig. S9). Importantly, β -costic acid

concentrations as low as 25 $\mu\text{g ml}^{-1}$ retained significant inhibitory activity in each case demonstrating that β -costic acid has the potential to function as wide-spectrum antifungal defense at low doses. To estimate *in vivo* roles, mature roots of greenhouse grown B73, Mo17 and 2 predominantly Mo17 IBM-NILs (Supplemental Fig. S9) were damaged and treated with either H_2O or H_2O containing spores of *F. verticillioides* and *F. graminearum* separately. Seven days later, the B73 inbred and the IBM-NIL (m050) harboring a *Zmtps21* pseudogene displayed significantly greater levels of disease as estimated by *Fusarium* DNA levels compared to Mo17 and the functional *ZmTps21* IBM-NIL (m065) (Fig. 6). Collectively, our results are consistent with ZmTps21 pathway products as mediators of antifungal defenses.

Discussion

Maize biochemicals either demonstrated or predicted to mediate insect and pathogen defense include diverse volatiles (Degenhardt, 2009; Degenhardt et al., 2009), benzoxazinoids (Frey et al., 2009; Ahmad et al., 2011; Meihls et al., 2013; Handrick et al., 2016), flavonoids and C-linked flavonoid glycosides (Meyer et al., 2007; Balmer et al., 2013; Casas et al., 2016), non-protein amino acids (Yan et al., 2015), oxylipins (Christensen et al., 2015; Borrego and Kolomiets, 2016), and non-volatile terpenoids (Schmelz et al., 2014). Among all biosynthetic classes, terpenoids are the most diverse structurally and in demonstrated breadth of ecological interactions mediated (Gershenzon and Dudareva, 2007). At the genome level, plants commonly possess mid-sized terpene synthase gene

372 families ranging from 14 to 70 members (Chen et al., 2011). More specifically, in
373 maize use of “terpene” as a keyword search in Phytozome
374 (<https://phytozome.jgi.doe.gov>) currently reveals more than 30 *Tps* gene models.
375 Collective efforts have resulted in the genetic, biochemical and ecological
376 characterization of approximately half of the maize enzymes encoded by *Tps*
377 with product specificity in the production of mono-, sesqui- and diterpenes
378 (Schnee et al., 2002; Kollner et al., 2004; Schnee et al., 2006; Kollner et al.,
379 2008; Degenhardt et al., 2009; Fu et al., 2016; Richter et al., 2016). Maize
380 terpene volatiles are often highly inducible following insect attack and aid in the
381 attraction of diverse natural enemies both above and below ground (Turlings et
382 al., 1990; Rasmann et al., 2005; Degenhardt et al., 2009). Oxygenated non-
383 volatile terpenoids can also accumulate and act as antifungal agents and insect
384 antifeedants (Schmelz et al., 2011). As part of this biochemical complexity, we
385 demonstrate that maize tissues are capable of accumulating both high levels of
386 the sesquiterpene olefin β -selinene and the corresponding non-volatile
387 oxygenated derivative termed β -costic acid. Intriguingly, β -costic acid is produced
388 in diverse aromatic and medicinal plants widely investigated for bioactive agents
389 driving antibiotic and anti-arthropod activities (Rao and Alvarez, 1981; Wu et al.,
390 2006; Katerinopoulos et al., 2011). Despite the widespread occurrence in nature,
391 *Tps* essential for the specific *in vivo* production of β -costic acid have not been
392 previously demonstrated *in planta*. We currently describe a maize β -selinene
393 synthase, termed ZmTps21, which is required for the inducible accumulation of
394 β -costic acid.

395 High tissue concentrations of β -costic acid were first detected in mature
396 field-collected roots of both sweet corn and Mo17 but appeared absent the B73
397 inbred. Use of complimentary mapping resources and the induced production of
398 β -costic acid as a qualitative trait demonstrated single a narrow QTL containing a
399 *Tps* gene candidate.

400 To examine the full-length Mo17 *ZmTps21* allele identified, heterologous
401 expression experiments were conducted in *E. coli* and protein extracts incubated
402 with FPP yielded β -selinene as the dominant volatile product. Based on
403 numerous inbred lines and predicted proteins from genome sequences
404 (Supplemental Fig. S5 and S6), the *in vitro* products of functional *ZmTps21* are
405 consistent with the endogenous presence of β -costic acid in all Mo17-like
406 *ZmTps21* lines and likewise an absence in all B73-like *Zmtps21* lines (Fig. 5).

407 Precursors of dominant biochemical defense pathways are commonly the
408 products of fully functional duplicate genes (Kollner et al., 2008; McMullen et al.,
409 2009); however, mGWAS mapping results (Fig. 2) and the exclusive presence of
410 β -costic acid (Fig. 5) in lines with full-length *ZmTps21* alleles collectively support
411 the existence of a single maize β -selinene synthase. At the enzymatic level, the
412 existence of a product specific β -selinene synthase was first reported in 1992
413 through the examination of *Citronella mitis* fruits; however, the specific *Tps* gene
414 responsible remains unknown (Belingheri et al., 1992). Acid-induced cyclization
415 of germacrenes can also yield selinenes making it highly probable that a
416 germacrene A synthase is responsible for the costol, costal, and costic acid
417 eudesmanes in costus root oil; however, it remains unknown if costus contains a

specific β -selinene synthase (de Kraker et al., 2001). Further elucidation of the β -costic acid pathway will require the discovery of a yet unresolved cytochrome P450 monooxygenase(s) performing sequential oxidations leading to the carboxylic acid. Characterized germacrene A oxidases from the Asteraceae drive the biosynthesis of germacrene A acid which following acid-induced rearrangement can yield blends that include β -costic acid (Nguyen et al., 2010). A related P450 that directly oxidizes β -selinene to yield β -costic acid is predicted to occur in maize yet remains unknown.

While numerous plants in nature constitutively make β -selinene in specific tissues and life stages, β -selinene is rarely detected in maize and has occurred only in the context of pathogen attack (Becker et al., 2014; Sowbhagya, 2014). Consistent with this observation we find *ZmTps21* transcripts largely undetectable in healthy control tissues or those experiencing simple mechanical damage (Fig. 5). In contrast, heat-killed *Fusarium* hyphae and a wide range of live fungal species elicit *ZmTps21* transcript accumulation and β -costic acid production. With conceptual similarities to the zealexin pathway, the elicitation kinetics of both *ZmTps21* transcripts and β -costic acid differ and are temporally behind those of *ZmTps6/11* and zealexins. Given the broader range of fungi species displaying β -costic acid mediated growth suppression at $25 \mu\text{g ml}^{-1}$ compared to similar assays using zealexins (Huffaker et al., 2011), it is possible that the *ZmTps21* pathway exists as an additional potent line of defense activated sequentially as maize plants experience sustained attack. If this hypothesis is true, related studies on maize disease resistance should note

biological roles for QTLs that include *ZmTps21*. Supportively, independent disease-related QTLs have been detected in broad regions spanning Bin 9.05 (Baumgarten et al., 2007; Berger et al., 2014). More specifically *ZmTps21* (GRMZM2G011151) has been identified as uniquely present in transcriptome analyses of resistant inbred lines associated with enhanced antifungal defenses (Lanubile et al., 2014). In an empirical assessment of the *in vivo* role of *ZmTps21* derived defenses, root experiments using B73, Mo17 and 2 Mo17 NILs support the suppression of both *F. graminearum* and *F. verticillioides* growth in lines carrying functional Mo17 *ZmTps21* alleles (Fig. 6). Most maize biochemical defenses likely function in the context of complex arrays of bioactive metabolites from numerous pathways. In this context, isogenic mutants in numerous inbred backgrounds would be an ideal and improved platform for the critical examination of *ZmTps21* mediated biological functions. While the present study does not accomplish this long-term goal, we provide a foundation and mechanistic justification for related research directions.

Curiously, of lines closely examined at the gene level, β -costic acid biosynthesis mediated by *ZmTps21* is associated with inbreds originating from U.S. breeding programs. In contrast, β -costic acid biosynthetic capacity is largely absent from more geographically diverse accessions. It is tempting to speculate that while the β -costic acid pathway is commonly absent due to a partial gene deletion, *ZmTps21* may have been maintained by positive selection during the breeding of U.S. maize lines. Western corn rootworm (WCR: *Diabrotica virgifera virgifera*) larvae exist as a candidate pest pressure known to devastate the roots

of temperate maize through below-ground herbivory and the promotion of secondary disease (Flint-Garcia et al., 2009; Gray et al., 2009). In growth chamber experiments, maize plants containing a functional *ZmTps21* allele produced both β -selinene and β -costic acid following damage by WCR larvae (Fig. 2). Consistent with a long-term association, unlike the generalist *D. balteata*, WCR larvae were not significantly affected in preference or performance by β -costic acid as a direct defense (Fig. 2). In this context, β -costic acid is likely to be more important in limiting the secondary spread of fungal pathogens promoted by root herbivory. However, while not specifically examined here, we speculate that root pools of β -selinene may serve as a volatile attractant to natural enemies of *Diabrotica* larvae such as entomopathogenic nematodes (Rasman et al., 2005; Degenhardt et al., 2009). This phenomena has been demonstrated in context of trace amounts of maize root caryophyllene elicited following WCR larval herbivory. More broadly, numerous root terpene volatiles can attract both entomopathogenic and phytopathogenic nematodes, a result that highlights complex tradeoffs in the deployment of rhizosphere signals (Ali et al., 2011).

In conclusion, our current study identifies the presence of numerous α/β -selinene derived metabolites in maize tissues following biotic stress. In numerous trials using select maize lines, β -selinene and β -costic acid exist as predominant *ZmTps21*-derived terpenoids produced following fungal elicitation, long-term root herbivory and combined field pressures. Antifungal assays using both *in vitro* and *in vivo* approaches support an antifungal defense role for *ZmTps21* pathway products. Root herbivores are likely to be additionally impacted given that β -

costic acid can reduce the performance of generalists such as *D. balteata* in controlled bioassays. The discovery of further immune-related biochemical traits is certain to continue given the extreme genetic diversity in maize highlighted by over 8000 representative transcript assemblies detectable in diverse germplasm that are absent from B73 (Hirsch et al., 2014). To fill existing voids highlighted by comparative genomics, the combined application of metabolomics, mapping, and *in vitro* biochemistry provides a useful approach to rapidly connect phenotypes with genotypes (Meihls et al., 2013; Handrick et al., 2016; Richter et al., 2016). Our current identification of ZmTps21 as a β -selinene synthase required for β -costic acid production adds to the foundational knowledge of useful maize biochemical pathways that can be intentionally combined for combating complex biotic pressures.

MATERIALS AND METHODS

Plant and Fungal Materials

Seeds of the Intermated B73×Mo17 (IBM) population of recombinant inbred lines (RILs) and the Goodman diversity panel (Flint-Garcia et al., 2005) were kindly provided by Dr. Peter Balint-Kurti (USDA-ARS, Raleigh, NC, USA) and Dr. Georg Jander (Boyce Thompson Institute, Ithaca, NY, USA) (Supplemental Table S2). The IBM-RILs and Goodman diversity panel (replicate 2) were planted at the Biology Field Station located on the University of California San Diego (UCSD) campus in La Jolla, CA, USA, during the summers of 2015 and 2016, respectively. Field challenged roots from B73, Mo17, hybrid sweet corn (*Zea*

mays var. Golden Queen; Southern States Cooperative, Inc. Richmond, VA,
 USA) and IBM RILs were recovered 70 days after planting, washed, frozen in
 liquid N₂, ground to a fine powder and ultimately used for genetic mapping.
 Seeds of indicated B73×Mo17 near-isogenic lines (provided by the Maize
 Genetic COOP Stock Center, Urbana, IL, USA), landrace inbreds (B73, Ki3,
 M37W, Ms71, M162W, CML247, Ki11, Mo18W, Hp301, TX303, Oh43, Oh7B,
 Ky21, Mo17, and W22; National Genetic Resources Program, Germplasm
 Resources Information Network, Ames, IA) were germinated in MetroMix 200
 (Sun Gro Horticulture Distribution, Inc.) supplemented with 14-14-14 Osmocote
 (Scotts Miracle-Gro) and grown in a greenhouse as previously described
 (Schmelz et al., 2009) (Supplemental Table S2). Fungal stock cultures of
Rhizopus microsporus (Northern Regional Research Laboratory stock no.
 54029), *Fusarium verticillioides* (Northern Regional Research Laboratory; NRRL
 stock no. 7415), *Fusarium graminearum* (NRRL stock no. 31084) *Aspergillus*
parasiticus (nor-1), and *Cochliobolus heterostrophus* were grown on V8 agar for
 12 days before the quantification and use of spores (Huffaker et al., 2011;
 Huffaker et al., 2013). Heat-killed *Fusarium venenatum* (strain PTA-2684)
 hyphae was commercially obtained (Monde Nissin Corporation Co.) and used
 safely for large-scale field mGWAS trials as a non-infectious elicitor lacking
 known *Fusarium* mycotoxins.

Genetic Mapping of *ZmTps21*

533 Using the presence of β -costic acid in necrotic tissues as a trait, the B73
534 *Zmtps21* locus was mapped using 216 IBM RIL lines (Lee et al., 2002) and
535 further supported using select B73xMo17 NILs (Eichten et al., 2011). Marker
536 data for the IBM RIL population were provided by Dr. Peter Balint-Kurti (USDA-
537 ARS, Raleigh, NC). Windows QTL Cartographer (Version 2.5;
538 <http://statgen.ncsu.edu/~shchwang/WQTLCart.htm>) was employed for metabolite
539 Quantitative Trait Locus (mQTL) analysis with composite interval mapping (CIM).
540 The WinQTLCart program was set as following: CIM program module=Model 6:
541 Standard Model, walking speed=1 cM, control marker numbers=5, window
542 size=10 cM, regression method=backward regression. Permutations (500) were
543 run to determine the $P < 0.05$ logarithm (base 10) of odds (LOD) significance
544 threshold (Churchill and Doerge, 1994). A list of RILs and NILs used for mapping
545 in this study are given in (Supplemental Table S2). In effort to confirm and
546 potentially refine the position of the mQTL identified using CIM, association
547 analyses were also conducted on the IBM RILs using the General Linear Model
548 (GLM) in TASSEL 5.0 (Bradbury et al., 2007) and the Unified Mixed Linear Model
549 (MLM) to effectively control for false positives arising from the differential
550 population structure and familial relatedness present in diversity panels (Yu et al.,
551 2006). Unlike diversity panels, differential population structure and familial
552 relatedness are not typically significant features in biparental RIL panels and thus
553 the GLM and MLM models were predicted to generate similar results in the IBM
554 RIL association analyses. Geneotypic data from imputed IBM RIL SNP markers
555 (July 2012 All Zea GBS final build; www.panzea.org) were used for association

analyses of root β -costic acid levels in the intermated B73 \times Mo17 (IBM) population. 173,984 SNP markers with < 20% missing genotypes and minor allele frequency (MAF) > 15% were used.

A metabolite based genome-wide association study (mGWAS) was conducted for elicited levels β -costic acid as a trait in the Goodman diversity panel (Flint-Garcia et al., 2005) using the Unified MLM in TASSEL 5.0 (Yu et al., 2006; Bradbury et al., 2007). Final analyses were conducted with the R package GAPIT (Zhang et al., 2010; Lipka et al., 2012), which involves EMMA (executed by R package) and Compressed MLM (CMLM) population parameters previously determined (P3D) to identify genomic regions putatively associated with the trait. GWAS analyses utilized a B73 Version 2 referenced HapMap consisting of 246,477 SNPs previously derived from an Illumina 50K array (Cook et al., 2012) and a genotyping by sequencing (GBS) strategy (Elshire et al., 2011) filtering less than 20% missing genotype data with minor allele frequencies (MAF) > 5% (Samayoa et al., 2015; Olukolu et al., 2016). The kinship matrix (K), estimated from 246,477 SNPs was used jointly with population structure (Q) to improve association analysis (VanRaden, 2008). All metabolite data were \log_2 transformed prior to statistical analysis to improve normality. The Quantile-Quantile plots and Manhattan plots were constructed in the R package qqman (<http://cran.r-project.org/web/packages/qqman>) (Turner, 2014).

Identification and Quantification of Metabolites

Unless otherwise stated, all maize tissue samples were rinsed with water, frozen in liquid N₂, ground to a fine powder in a mortar and stored at -80 C for further analyses. For Vapor Phase Extraction (VPE) based sample preparation, 50 mg aliquots were first weighed, solvent extracted in bead homogenizer, and derivatized using trimethylsilyldiazomethane as previously described (Schmelz et al., 2004; Schmelz et al., 2011). GC-MS analysis was conducted using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 mass selective detector (interface temp, 250°C; mass temp, 150°C; source temp, 230°C; electron energy, 70 eV). The GC was operated with a DB-35MS column (Agilent, Santa Clara, USA, 30 m x 0.25 mm x 0.25 µm). The sample was introduced as a splitless injection with an initial oven temperature of 45°C. The temperature was held for 2.25 min, then increased to 300°C with a gradient of 20°C min⁻¹, and held at 300°C for 5 min. GC/EI-MS based quantification of β-costic acid was based upon the slope of an external standard curve constructed from β-costic acid (Ark Pharm, #AK168379) spiked into 50 mg aliquots of frozen powdered untreated maize stem tissues which were then processed using VPE (Schmelz et al., 2004). In representative samples analyzed by GC/EI-MS, β-costol was identified based 99% EI match within the Robert P. Adams essential oil MS library (Allured Books). While not previously detected in maize, β-costol is an anticipated intermediate in samples rich in both β-selinene and β-costic acid.

For headspace recovery of ZmTps21 enzyme products by solid phase micro-extraction (SPME), fibers containing 100 µm polydimethylsiloxane (SUPELCO, Belafonte, PA, USA) were placed into reaction vials for 60 min

incubations at 30°C and then introduced into the GC injector for the analyses of the adsorbed reaction products. GC-MS analyses conducted on SPME samples utilized a splitless injection, a DB-5MS column (Agilent, Santa Clara, USA, 30 m x 0.25 mm x 0.25 µm) and an initial oven temperature of 80°C. The temperature was held for 2 min, then increased to 240°C with a gradient of 7°C min⁻¹, and further increased to 300°C with a gradient of 60°C min⁻¹ and a hold of 2 min. Precise instrument settings of the Agilent 5973 mass selective detector were identical to those stated above used for plant samples. For GC-MS analysis with a cooler injector, the injector temperature was reduced from 240°C to 150°C.

Volatiles emitted from elicited stems and naturally challenged roots of field grown plants were collected by passing purified air over the tissue samples at 600 ml min⁻¹ and trapped on inert filters containing 50 mg HayeSep® Q (80-100 µm mesh) polymer adsorbent (Sigma-Aldrich, St. Louis, MO, USA). Individual samples were then eluted with 150 µl methylene chloride, and analyzed by GC coupled with flame ionization detector (FID) as previously described (Schmelz et al., 2001). β-selinene and related volatiles were quantified by GC-FID using the slope of an external standard curve of (*E*)-β-farnesene. Select samples were analyzed by GC/EI-MS to confirm individual peak identities of representative replicates. This included the comparison of retention times with authentic standards and by comparison of mass spectra with Wiley, National Institute of Standards and Technology and the Adams libraries.

To ensure maximal independence of the second GWAS replicate that was grown in the field, analytical conditions utilized LC-MS instead of GC-MS.

624 Reacted stem tissues were first ground to a fine powder with liquid N₂ and
625 weighed out in 50 mg aliquots. Tissue samples were sequentially and additively
626 bead homogenized in 1) 100 µl 1-propanol: acetonitrile: formic acid (1:1:0.01), 2)
627 250 µl acetonitrile: ethyl acetate (1:1), and 3) 100 µl of H₂O. Each combined
628 sample consisted of a co-miscible acidified solvent mixture of primarily 1-
629 propanol: acetonitrile: ethyl acetate: water in the approximate proportion of
630 11:39:28:22 which was then centrifuged at 15,000 rpm for 20 min. Approximately
631 150 µl the particulate free supernatant was carefully removed for LC/MS
632 automated sample analyses utilizing 5 µl injections. The LC consisted of an
633 Agilent 1260 Infinity series HiP Degasser (G4225A), 1260 binary pump
634 (G1312B), and a 1260 autosampler (G1329B). The binary gradient mobile phase
635 consisted of 0.1% formic acid in H₂O (solvent A) and 0.1% formic acid in MeOH
636 (solvent B). Analytical samples were chromatographically separated on a Zorbax
637 Eclipse Plus C18 Rapid Resolution HD column (Agilent: 1.8 µm, 2.1 x 50 mm)
638 using a 0.35 ml min⁻¹ flow rate. The mobile phase gradient was: 0–2 min, 5% B
639 constant ratio; 3 min, 24% B; 18 min, 98% B, 25 min, 98% B, and 26 min 5% B
640 for column re-equilibration before the next injection. Eluted analytes underwent
641 electrospray ionization (ESI) via an Agilent Jet Stream Source with thermal
642 gradient focusing using the following parameters: nozzle voltage (500 V), N₂
643 nebulizing gas (flow 12 l min⁻¹, 55 psi, 225°C) and sheath gas (350°C, 12 l min⁻¹).
644 The transfer inlet capillary was 3500V and both MS1 and MS2 heaters were at
645 100°C. Negative ionization [M-H]⁻ mode scans (0.1 amu steps, 2.25 cycles s⁻¹)
646 from *m/z* 100 to 1000 were acquired. After considerable unsuccessful attempts to

optimize parameters required to obtain meaningful daughter ion fragments from β -costic acid, analyses relied exclusively on the native parent $[M-H]^-$ ion m/z 233 and stable retention time of 16.65 min separated from established maize zealexins. Quantification utilized an external standard curve of β -costic acid (Ark Pharm, #AK168379, Libertyville, IL, USA) analyzed under identical conditions.

Controlled Maize Elicitation Assays

Controlled maize elicitation assays used 30-40 day old greenhouse plants grown in 1-l plastic pots or in the case of the Goodman diversity panel (second replicate) field grown plants. Plants in damage-related treatment groups were slit in the center, spanning both sides of the stem, with a surgical scalpel that was pulled 8–10 cm upward to create a parallel longitudinal incision. The treatment spanned the upper nodes, internodes, and the most basal portion of unexpanded leaves. All fungal spore inoculations ($1 \times 10^7 \text{ ml}^{-1}$) treatments were performed in 100 μl of H_2O . For experiments involving stem elicitation with heat-killed *Fusarium* hyphae, crude material was homogenized in a Waring blender at maximum speed for 3 min in the presence of additional H_2O at 20-30% (WT/WT) to create a thick smooth paste. Approximately 500 μl of crude elicitor was introduced into each slit stem followed by sealing the site with clear plastic packing tape to minimize desiccation of the treated stem tissues. For each individual experiment details relating to specific tissues, biological replications and harvest time points are noted in the figures and captions.

For the assay of plant responses to long-term western corn rootworm (WCR; *Diabrotica virgifera virgifera*) herbivory, seeds of an IBM line carrying a functional *ZmTps21* gene (IBM-RIL-0287) were grown in 946 ml DM32R cups (Dart Container Corporation, Mason, MI, USA) filled with greenhouse potting media and fertilized following Gassmann et al. (Gassmann et al., 2011). Seeds were planted 1 month prior to WCR inoculation and maintained at 23°C – 28°C in a greenhouse with supplemental daylight balanced illumination on a 16:8 (L:D) photoperiod. Plants were watered daily as needed to prevent saturated soil conditions. Inoculation and care of ≥V5-V6 stage (Abendroth, 2011) treated plants followed from Gassmann et al. (Gassmann et al., 2011). Cups were inoculated with n=10 neonate WCR larvae (obtained from USDA-ARS-NCARL; Brookings, SD, USA) and held in an incubator at 24°C with 40%-60% RH and watered sparingly as needed to minimize pot flooding. The experiment utilized 4 replicates per treatment. After 17 d, 1 g samples of insect attacked and healthy root tissues were collected from the plants, frozen on dry ice and stored for chemical analyses.

***Diabrotica* Species Preference and Performance Assays**

For studies on preference and performance, WCR eggs and *Diabrotica balteata* (LeConte) eggs were obtained from USDA-ARS-NCARL (Brookings, SD, USA) and Syngenta (Syngenta Crop Protection AG, CHE), respectively. All larvae were reared on the roots of germinating maize seedlings until use. For both *Diabrotica* species, 3rd instar larvae were used for all experiments. The

performance of *D. virgifera* and *D. balteata* larvae was evaluated by placing one pre-weighed larva into individual solo cups (Bioserv, Newark, DE, USA) containing moist filter paper and a 60 mg crown root section from the B73 inbred. Crown roots were covered with 50 μ L β -costic acid in EtOH: H₂O (15 %: 85 %) to create a final tissue concentration of 100 μ g g⁻¹ FW. Control roots were similarly treated with 50 μ L EtOH: H₂O (15 %: 85 %). Larval growth was determined after 48 h. The preference of the root herbivores given a choice between control and β -costic acid complemented roots was evaluated in 9 cm dia. petri dishes (Greiner Bio-One GmbH, Frickenhausen, DE). Root tissue treatments followed from the performance experiment. One root of each treatment was placed in the petri dishes. Five larvae were introduced in between the two root sections and larvae feeding behavior was recorded at 0.5, 1, 2, 3 and 4 h after start of the trials.

RNA Isolation and qRT-PCR

Total RNA was isolated with TRIzol (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized with the RETROscript reverse transcriptase kit (Ambion, Waltham, MA, USA) using random decamer primers. qRT-PCR was performed using Power SYBR Green Master mix (Applied Biosystems, Waltham, MA, USA), and 250 nM primers on a Bio-Rad CFX96TM Real-Time PCR Detection System. Mean cycle threshold values of triplicate reactions were normalized to EF-1 α (GenBank accession no. AF136829) (Huffaker et al., 2011). Fold-change calculations were performed

using the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). qRT-PCR primers used in the current study are listed (Supplemental Table S1)

Isolation of *ZmTps21* cDNA from Mo17

Total RNA was isolated as described above and subjected to TURBO™ DNA-free™ treatment (Ambion) followed by total RNA purification with RNeasy® Mini protocol for RNA cleanup (Qiagen, Hilden, GR). Approximately 1 µg of an equally mixed RNA pool from Mo17 meristem tissues elicited with heat killed *Fusarium* hyphae collected at different time points (8, 24, 32, and 48 h) was used for the construction of 5'- or 3'-RACE cDNA library with SMARTer RACE 5'/3' Kit (Clontech, Mountain View, CA, USA) in accordance with the manufacturers' protocol. The 5'-end of B73-*Zmtps21* was used to design primers for PCR amplification of the Mo17 *ZmTps21* gDNA. A DNA fragment, which was larger than the one from B73 on the agarose gel, was amplified using primers 5'-TGTGAACCAACAAAGCAAGGC-3' and 5'-GAGCTCACCAATCATAGCCTC-3' cloned and sequenced. Based on the conserved sequences between B73 and Mo17, primers were designed to amplify of the 3' and 5' ends *via* rapid amplification of cDNA ends (RACE) (Clontech) from 5'/3' cDNA libraries of *Fusarium* elicited meristems of Mo17. The complete cDNA sequence of the Mo17 functional *ZmTps21* was amplified with the primers Mo17 *ZmTPS21F* (5'-ATGGATGGTGATATTGCTGCCG-3') and Mo17 *ZmTps21R* (5'-TCAGGCACACGGCTTGAGG-3') from the Mo17 5'-RACE cDNA library. Primers used to amplify *ZmTps21* genomic DNA from B73, W22, CML247 and other diverse inbred lines (Ki3, M37W, MS71, M162W, Ki11, Mo18W, HP301, TX303,

OH43, Oh7B, KY21, Mo17) are listed (Supplemental Table S1). Corresponding unpublished sequences were deposited in GenBank with following accession numbers (MF614104, MF614105, MF614106, MF614107, MF614108, MF614109, MF614110, MF614111, MF614112, MF614113, MF614114, MF614115).

Assay for Terpene Synthase Activity

The complete open reading frame of Mo17 *ZmTps21* was amplified with the primers Mo17 *ZmTps21*-fwd (CACCATGGATGGTGATATTGCTGCCG) and Mo17 *ZmTps21*-rev (TCAGGCACACGGCTTGAGGAAC) and the resulting PCR fragment was cloned into the vector pET100/D-TOPO[®] (Invitrogen, Carlsbad, CA, USA). Sequencing of several clones revealed intact Mo17 *ZmTps21* and two cloning artifacts with altered 3' ends. For heterologous expression in *E. coli*, the plasmids were introduced into the strain BL21 Codon Plus (Invitrogen, Carlsbad, CA, USA). Expression was induced by addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 1 mM. The cells were collected by centrifugation at 4,000g for 6 min, and disrupted by a 4 × 30 sec treatment with a sonicator in chilled extraction buffer (50 mM MOPS, pH 7.0, with 5 mM MgCl₂, 5 mM sodium ascorbate, 0.5 mM PMSF, 5 mM dithiothreitol and 10% v/v glycerol). The cell fragments were removed by centrifugation at 14,000 g, and the supernatant was desalted into assay buffer (10 mM MOPS, pH 7.0, 1 mM dithiothreitol, 10% v/v glycerol) by passage through a Econopac 10DG column (BioRad, Hercules, CA, USA). Enzyme assays were performed in a Teflon[®]-

sealed, screw-capped 1 ml GC glass vial containing 50 µl of the bacterial extract and 50 µl assay buffer with 10 µM (*E,E*)-FPP and 10 mM MgCl₂. SPME fiber sample enrichment of adsorbed reaction products and analyses by GC/MS is detailed above in “Identification and Quantification of Metabolites”.

Bioassays of *in vitro* and *in vivo* β-costic acid activity as an antifungal agent

Maize antifungal assays using purified β-costic acid (Ark Pharm, #AK168379) were performed using the Clinical and Laboratory Standards Institute M38-A2 guidelines as previously detailed (Schmelz et al., 2011). In brief, a 96-well microtiter plate-based method using a Synergy4 (BioTech Instruments, Inc.) reader was used to monitor fungal growth at 30 °C in broth media through periodic measurements of changes in OD at 600 nm. Each well contained 200 µl of initial fungal inoculum (2.5×10^4 conidia ml⁻¹) with 0.5 µl of either pure DMSO or DMSO containing dilutions of β-costic acid.

For the mature root infection assays with *Fusarium* pathogens, individual maize plants were greenhouse grown in separate 10-liter pots and supplemented with 14-14-14 Osmocote (Scotts Miracle-Gro) fertilizer. In effort to closely parallel our observations from mature field roots and minimize the invasiveness of belowground treatments, we limited our selection to large nodal roots (≥2mm dia.) containing 1st order lateral roots that were visually apparent and easily accessed following the temporary removal of the pot. Spanning a length of 8 cm,

786 at 1 cm intervals selected nodal roots were punctured with a blunt ended circular
 787 steel pin (0.6mm dia) creating a total of 9 punctures. Divided across the 9 wound
 788 sites per nodal root and depending on treatment, 100 μ ls of either H₂O or 1×10^7
 789 conida mL⁻¹ of either *F. verticillioides* (*F.v.*) or *F. graminearum* (*F.g.*) were
 790 applied. Treatments were limited to exposed roots growing along the outer-edge
 791 of the soil in close contact with the vertical wall of the plastic pot. Following
 792 treatments, plants were carefully placed back into the pots for 7 days. For each
 793 line grown, namely B73, m050, Mo17 and m065, 3 treatments and 4 replicates
 794 were performed ($4 \times 4 \times 3 = 48$ plants). For determination of the fungal biomass,
 795 inoculated and damaged roots were collected 7 days after fungal inoculation.
 796 Total genomic DNA was extracted from the infected roots and subjected to real
 797 time qPCR using the *F. graminearum*-specific primers for a deoxynivalenol
 798 mycotoxin biosynthetic gene (*FgTri6*) and *F. verticillioides* specific primers for a
 799 calmodulin (*FvVER1*) gene (Mule et al., 2004; Horevaj et al., 2011) (*SI Appendix*,
 800 Table S1). The amount of pathogen DNA relative to plant DNA was estimated by
 801 qRT-PCR. Plant DNA quantification utilized a conserved genomic sequence of
 802 *ZmTps21/Zmtps21* DNA shared between B73 and Mo17 using forward (gTps21-
 803 F, GCAGATGTGTTTCGACAAGTTCC) and reverse (gTps21 R-
 804 TTACCTGCAGATTTCTCTAAGCTCTC) primers with calculated amplification
 805 efficiencies of 102.65-102.89% between inbreds (Supplemental Table S1).
 806 Relative amounts of fungal DNA were calculated by the $2^{-\Delta\Delta C_t}$ method,
 807 normalized to a conserved genomic sequence of *ZmTps21/Zmtps21* DNA shared
 808 between B73 and Mo17.

809

810 **Statistical Analyses**

811 ANOVAs were performed on the quantified levels of terpenoids, qRT-PCR
812 transcripts, fungal growth and levels of fungal DNA. Treatment effects were
813 investigated when the main effects of the ANOVAs were significant ($P < 0.05$).
814 Tukey tests were used to correct for multiple comparisons between control and
815 treatment groups. The short-term preference and 2-d performance of *Diabrotica*
816 larvae on roots, with and without additional β -costic acid, were analyzed with one
817 sample t-tests and two-way ANOVA using SigmaPlot 13.0 (Systat Software Inc,
818 San Jose, CA, USA), respectively.

819

820 **Supplemental Materials**

821 **Figure S1.** α/β -selinene derived oxidative products, β -costol, β -costal, α -costic
822 acid and β -costic acid coexist as a network of maize metabolites.

823 **Figure S2.** Replicated and comparative association analyses confirm detection
824 of *ZmTps21* as a gene candidate involved in β -costic acid biosynthesis.

825 **Figure S3.** Confirmation of the locus identified by combined linkage and
826 association mapping based on β -costic acid levels using B73 and Mo17 near
827 isogenic lines (NILs).

828 **Figure S4.** Sequence comparison of Mo17 *ZmTps21* with other plant terpene
829 synthases known to catalyze the protonation of neutral reaction intermediates.

830 **Fig. S5.** *ZmTps21* gene structure and sequence polymorphisms across
831 numerous diverse inbred lines support the occurrence of a common and
832 conserved B73-like mutation.

Figure S6. Deduced amino acid sequence comparison of ZmTps21 across select maize inbred lines.

Figure. S7. C-terminal modifications in Mo17 ZmTps21 support an influential role in the protonation of germacrene A as putative reaction intermediate

Figure S8. Germacrene A is minor yet detectable product of Mo17 ZmTps21 and is converted to β -elemene during GC injection at 240°C.

Figure S9. ZmTps21 derived products inhibit fungal growth at physiologically relevant concentrations *in vitro* and can be assessed *in vivo* using IBM near isogenic lines (NILs).

Table S1. Primers used for qRT-PCR analysis and sequencing *ZmTps21* genomic DNA

Table S2. Maize lines specifically used to identify *ZmTps21*

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Figure Legends

Figure 1. β -selinene and β -costic acid can occur as major components of maize roots in field grown plants. Visibly (A) infected or (B) healthy field collected sweet corn (var. Golden Queen) root samples following trimethylsilyldiazomethane derivatization of carboxylic acids to corresponding methyl esters. Labeled peaks in representative GC/EI-MS total ion chromatograms (TIC) include: 1, β -selinene; 2, α -selinene (shoulder); 3, β -costic acid; 4, zealexin A1; and 5, zealexin B1. The presence of common fatty acids, namely palmitic acid and steric acid, are unchanged in healthy root tissues and directly labeled for reference. Corresponding EI spectra (m/z) of (C) β -selinene, (D) α -selinene, and (E) β -costic acid methyl ester from maize field collected roots. (F) Proposed α/β -costic acid biosynthetic pathway in maize starting from farnesyl diphosphate (FPP).

Figure 2. β -selinene can exist as a dominant elicited volatile and the pathway product β -costic acid can reduce herbivore performance. Representative GC-FID traces of volatile emissions collected from live roots of field grown maize lines (A) B73, (B) Mo17 and (C) IBM-RIL 0287 20 days after pollination. (D) Average ($n = 4$, \pm SEM) quantity ($\mu\text{g } 12 \text{ h}^{-1} \text{ g}^{-1} \text{ DW}$) of β -selinene volatiles emitted from respective maize roots. Representative GC-FID traces of emitted volatiles collected from living (E) control B73, (F) *C. heterostrophus*-infected B73, (G) control Mo17 and (H) Mo17 *C. heterostrophus*-infected stems. (I) Average ($n = 4$, \pm SEM) quantity ($\text{ng cm}^{-2} \text{ h}^{-1}$) of β -selinene emitted as a volatile from the stems of 5-week-old plants following damage and treatment with H_2O (Dam) or with $100 \mu\text{l}$ of 1×10^7 spores *C. heterostrophus* (*C.h.*). Within plots D and I, different letters (a–c) represent significant differences (All ANOVA P s < 0.05 ; Tukey test corrections for multiple comparisons: $P < 0.05$). (J) Average ($n = 4$, \pm SEM) root tissue concentrations ($\mu\text{g g}^{-1} \text{ FW}$) of β -selinene and β -costic acid levels in the roots of IBM-RIL-0287 following 17 days of either no treatment (Ctr) or herbivory by western corn rootworm (WCR) (*Diabrotica virgifera virgifera*) larvae (Student's t-test; one-tailed distribution, equal variance). (K) Average WCR ($n = 18$, \pm SEM) and *Diabrotica balteata* ($n = 57$, \pm SEM) preference over 4 h for excised maize roots treated with either EtOH: H_2O (15:85) alone (Control) or the same solution containing β -costic acid to achieve a root tissue concentration of $100 \mu\text{g g}^{-1} \text{ FW}$. Each replicate (n) consisted of assays with 5 individual 3rd instar larvae where distributions were measured at 30, 60, 90, 120, 180, 240 min and collectively averaged (one sample t-test, P s > 0.05). (L) Average ($n \geq 5$, \pm SEM) performance (% relative weight gain) of 3rd instar WCR and *D. balteata* larvae over 2 days of feeding on root tissues with (+) and without (-) additions of β -costic acid as described in the preference study (two-way ANOVA $P < 0.05$).

Figure 3. Combined linkage and association mapping identifies *ZmTps21* as a candidate β -selinene synthase. (A) Major mQTL for β -costic acid production detected on chromosome 9 by composite interval mapping (CIM) using IBM recombinant inbred lines (RILs). (A-insert) Comparative association analysis of the IBM-RIL β -costic acid levels using the General Linear Model (GLM) and 173,984 SNPs. The most statistically significant SNP is located at 127,854,265 on Chromosome 9 (B73 RefGen_v2) with a dashed line denoting the 5% Bonferroni correction. (B) Quantile-quantile plot for association analysis of β -costic acid levels in the Goodman diversity panel. (C) Manhattan plot of the association analysis (MLM) of β -costic acid levels in replicate 1 of the Goodman diversity panel following 3 days of fungal elicitation. Dashed line denotes the 5% bonferroni-corrected threshold for 246,477 SNP markers with the most statistically significant SNP located at 127,858,963 (B73 RefGen_v2) on Chromosome 9. (D) Location of the candidate gene *ZmTps21* on the physical map supported by both linkage analysis and association analysis. (E) Fine-mapping with IBM near-isogenic lines (NILs); B73 and Mo17 chromosomal segments are represented by blue and red, respectively. β -costic acid chemotypes of IBM-NILs are indicated as GC/EI-MS traces ($m/z = 233$). (F) Agarose gel PCR amplified products demonstrate a cDNA length polymorphism between B73 *Zmtps21* and Mo17 *ZmTps21* candidates. (G) Diagrammatic structures of B73 *Zmtps21* and Mo17 *ZmTps21* genes based on sequencing. Exons and introns are denoted as rectangular bars and as black lines, respectively. Open rectangle indicates the missing B73 genomic DNA and relative position of encoded conserved RXR and DDXXD motifs terpene cyclase activity.

Figure 4. Mo17 *ZmTps21* encodes a functional β -selinene synthase. (A) Mo17 *ZmTps21* was in *Escherichia coli* and the resulting protein extract was incubated with (*E,E*)-farnesyl diphosphate (FPP). Mo17 *ZmTps21* products were collected using solid-phase microextraction and analyzed by GC/MS revealing (2) β -selinene as the dominant product with lower yet detectable levels of (1) β -elemene (germacrene A rearrangement product) and (3) α -selinene. (B) Celery fruit essential oil was used as a natural product standard for β -selinene/ α -selinene (9:1).

Figure 5. *ZmTps21* transcripts are elicited by diverse fungi and precede β -costic acid accumulation detectable in diverse maize lines. Average ($n = 4$; \pm SEM) Mo17 (A) *ZmTps21* (B) β -costic acid (C), *ZmTps6/11* and (D) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations ($\mu\text{g g}^{-1}$ FW) in intact control stems (Con) or those damaged and treated with either H₂O (Dam), or a heat-killed *Fusarium* elicitor (F.E.) hyphae preparation after 1, 2, or 4 days. Average ($n = 4$; \pm SEM) Mo17 (E) *ZmTps21* (F) β -costic acid (G), *ZmTps6/11* and (H) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations ($\mu\text{g g}^{-1}$ FW) in intact control stems (Con) or those damaged and treated with either 100 μl of H₂O (Dam) alone or spore suspensions ($1 \times 10^7 \text{ mL}^{-1}$) of *R. microsporus* (*R.m.*), *A. parasiticus* nor-1 (*A.p.*), *F. verticillioides* (*F.v.*), or *C. heterostrophus* (*C.h.*) and harvested at 2 and 4 days for transcripts and metabolites, respectively. (I) Average ($n = 4$, \pm SEM) β -costic acid concentrations ($\mu\text{g g}^{-1}$ FW) in the scutella of 10-d-old maize seedlings from 15 inbred maize lines and mature field collected roots displaying necrosis. Hybrids include sweet corn (var. Golden Queen; GQ) and IBM-RIL0287. Within plots, different letters (a-e) represent significant differences (all ANOVA $P < 0.05$; Tukey test corrections for multiple comparisons: $P < 0.05$).

Figure 6. *ZmTps21* derived products inhibit *Fusarium* fungi *in vitro* and correspond with improved disease resistance *in vivo*. Average ($n = 8$, \pm SEM) fungal growth estimates (600 nm OD) of (A) *F. verticillioides*, and (B) *F. graminearum*, in liquid media in the presence of β -costic acid at 0 (\circ), 25 (\bullet), and 100 (Δ) $\mu\text{g mL}^{-1}$. Average ($n = 4$, \pm SEM) ratio of fungal DNA / plant DNA levels present in maize roots 7 days after damage and inoculation with 100 μl s of either H₂O or 1×10^7 conidia mL^{-1} of (C) *F. verticillioides* and (D) *F. graminearum* in B73, Mo17 and IBM-NILs harboring active (+; m065) and inactive (-; m050) alleles of *ZmTps21*. Within plots, different letters (a–c) represent significant differences (All ANOVA $P < 0.05$; Tukey test corrections for multiple comparisons: $P < 0.05$).

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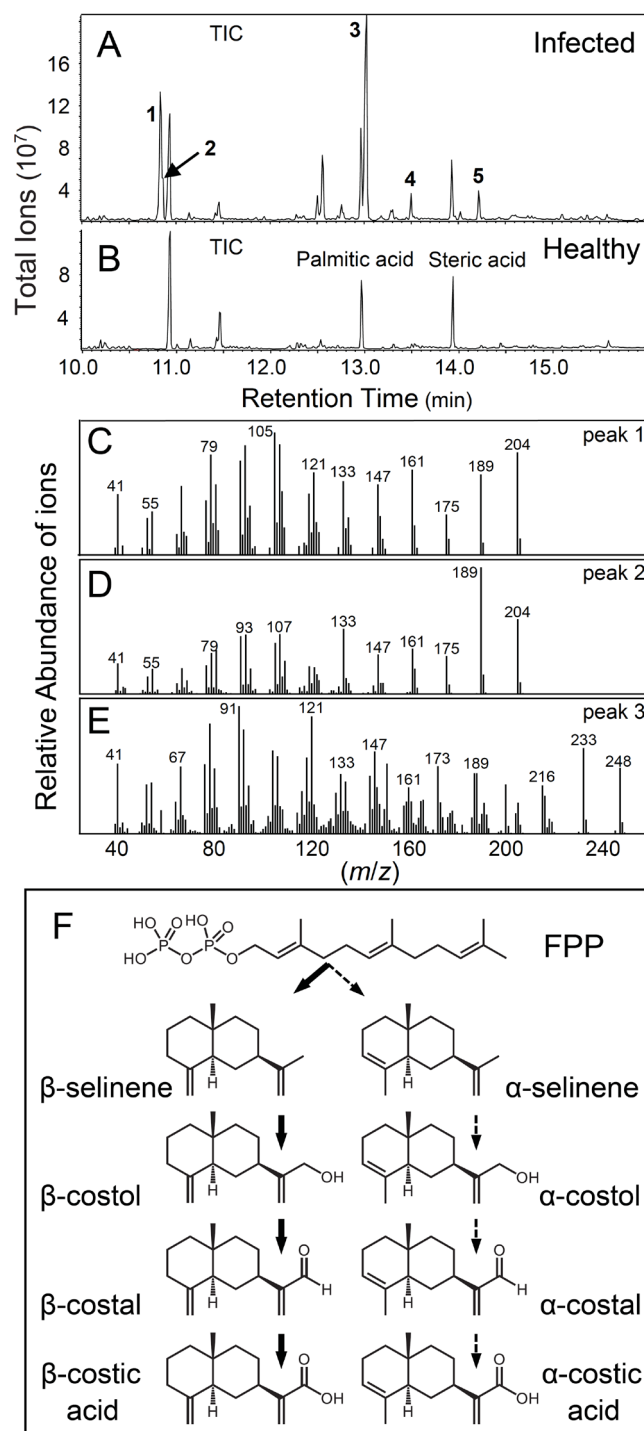


Figure 1. β -selinene and β -costic acid can occur as major components of maize roots in field grown plants. Visibly (A) infected or (B) healthy field collected sweet corn (var. Golden Queen) root samples following trimethylsilyldiazomethane derivatization of carboxylic acids to corresponding methyl esters. Labeled peaks in representative GC/EI-MS total ion chromatograms (TIC) include: 1, β -selinene; 2, α -selinene (shoulder); 3, β -costic acid; 4, zealexin A1; and 5, zealexin B1. The presence of common fatty acids, namely palmitic acid and steric acid, are unchanged in healthy root tissues and directly labeled for reference. Corresponding EI spectra (m/z) of (C) β -selinene, (D) α -selinene, and (E) β -costic acid methyl ester from maize field collected roots. (F) Proposed α/β -costic acid biosynthetic pathway in maize starting from farnesyl diphosphate (FPP).

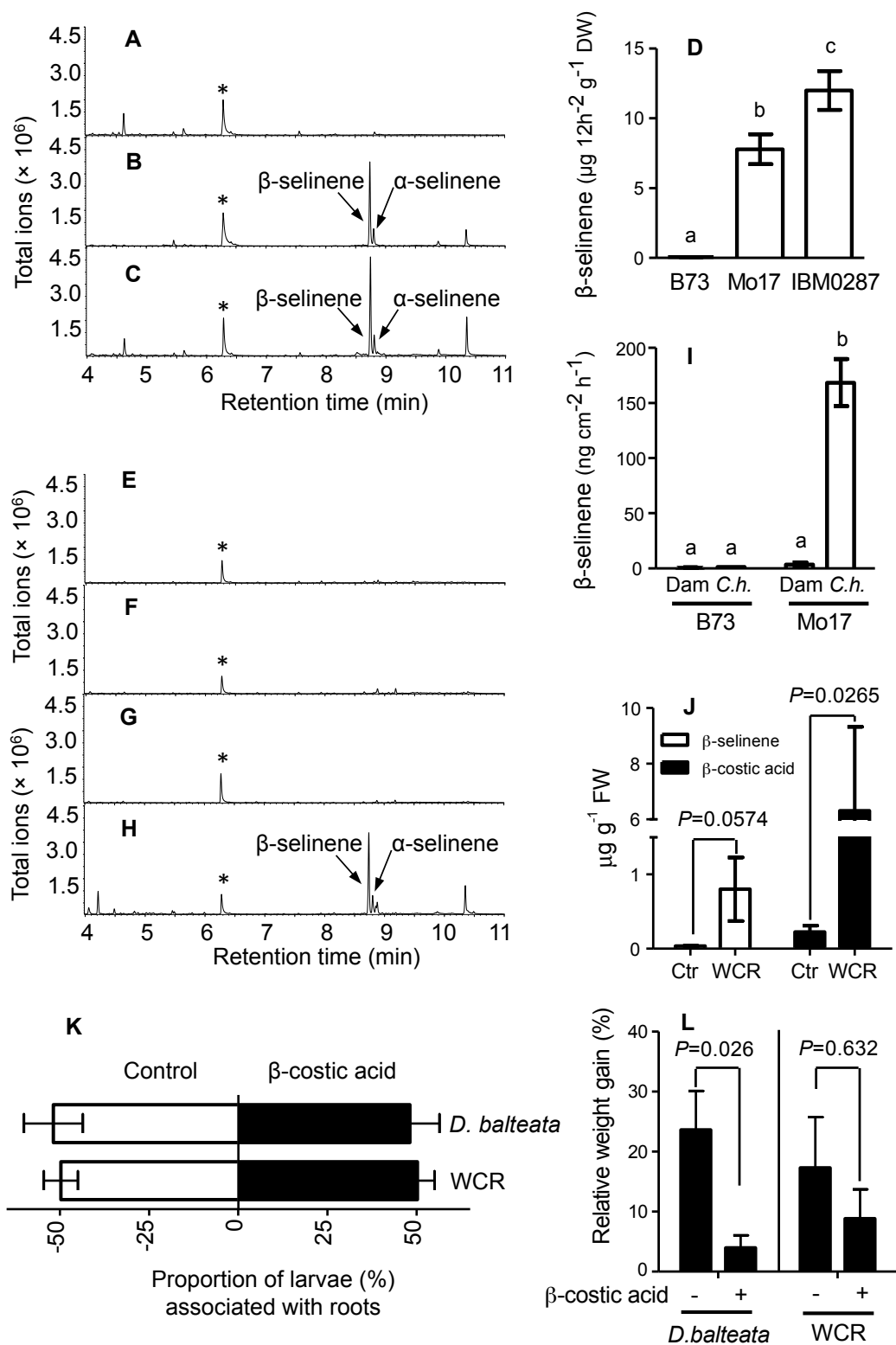


Figure 2. β -selinene can exist as a dominant elicited volatile and the pathway product β -costic acid can reduce herbivore performance. Representative GC-FID traces of volatile emissions collected from live roots of field grown maize lines (A) B73, (B) Mo17 and (C) IBM-RIL 0287 20 days after pollination. (D) Average ($n = 4$, \pm SEM) quantity ($\mu\text{g } 12 \text{ h}^{-1} \text{ g}^{-1} \text{ DW}$) of β -selinene volatiles emitted from respective maize roots. Representative GC-FID traces of emitted volatiles collected from living (E) control B73, (F) *C. heterostrophus*-infected B73, (G) control Mo17 and (H) Mo17 *C. heterostrophus*-infected stems. (I) Average ($n = 4$, \pm SEM) quantity ($\text{ng cm}^{-2} \text{ h}^{-1}$) of β -selinene emitted as a volatile from the stems of 5-week-old plants following damage and treatment with H_2O (Dam) or with $100 \mu\text{l}$ of 1×10^7 spores *C. heterostrophus* (*C.h.*). Within plots D and I, different letters (a–c) represent significant differences (All ANOVA P s < 0.05 ; Tukey test corrections for multiple comparisons: $P < 0.05$). (J) Average ($n = 4$, \pm SEM) root tissue concentrations ($\mu\text{g g}^{-1} \text{ FW}$) of β -selinene and β -costic acid levels in the roots of IBM-RIL-0287 following 17 days of either no treatment (Ctr) or herbivory by western corn rootworm (WCR) (*Diabrotica virgifera virgifera*) larvae (Student's t-test; one-tailed distribution, equal variance). (K) Average WCR ($n = 18$, \pm SEM) and *Diabrotica balteata* ($n = 57$, \pm SEM) preference over 4 h for excised maize roots treated with either EtOH: H_2O (15:85) alone (Control) or the same solution containing β -costic acid to achieve a root tissue concentration of $100 \mu\text{g g}^{-1} \text{ FW}$. Each replicate (n) consisted of assays with 5 individual 3rd instar larvae where distributions were measured at 30, 60, 90, 120, 180, 240 min and collectively averaged (one sample t-test, P s > 0.05). (L) Average ($n \geq 5$, \pm SEM) performance (% relative weight gain) of 3rd instar WCR and *D. balteata* larvae over 2 days of feeding on root tissues with (+) and without (-) additions of β -costic acid as described in the preference study (two-way ANOVA $P < 0.05$).

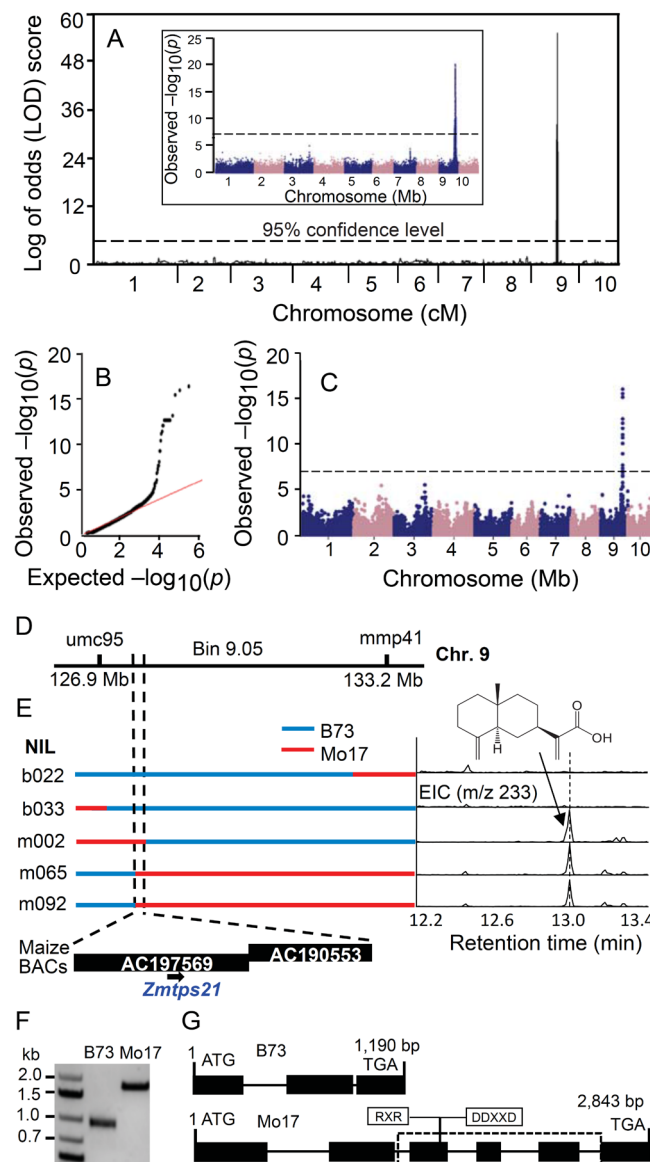


Figure 3. Combined linkage and association mapping identifies *ZmTps21* as a candidate β -selinene synthase. (A) Major mQTL for β -costic acid production detected on chromosome 9 by composite interval mapping (CIM) using IBM recombinant inbred lines (RILs). (A-insert) Comparative association analysis of the IBM-RIL β -costic acid levels using the General Linear Model (GLM) and 173,984 SNPs. The most statistically significant SNP is located at 127,854,265 on Chromosome 9 (B73 RefGen_v2) with a dashed line denoting the 5% Bonferroni correction. (B) Quantile-quantile plot for association analysis of β -costic acid levels in the Goodman diversity panel. (C) Manhattan plot of the association analysis (MLM) of β -costic acid levels in replicate 1 of the Goodman diversity panel following 3 days of fungal elicitation. Dashed line denotes the 5% bonferroni-corrected threshold for 246,477 SNP markers with the most statistically significant SNP located at 127,858,963 (B73 RefGen_v2) on Chromosome 9. (D) Location of the candidate gene *ZmTps21* on the physical map supported by both linkage analysis and association analysis. (E) Fine-mapping with IBM near-isogenic lines (NILs); B73 and Mo17 chromosomal segments are represented by blue and red, respectively. β -costic acid chemotypes of IBM-NILs are indicated as GC/EI-MS traces ($m/z = 233$). (F) Agarose gel PCR amplified products demonstrate a cDNA length polymorphism between B73 *ZmTps21* and Mo17 *ZmTps21* candidates. (G) Diagrammatic structures of B73 *ZmTps21* and Mo17 *ZmTps21* genes based on sequencing. Exons and introns are denoted as rectangular bars and as black lines, respectively. Open rectangle indicates the missing B73 genomic DNA and relative position of encoded conserved RXR and DDXXD domains.

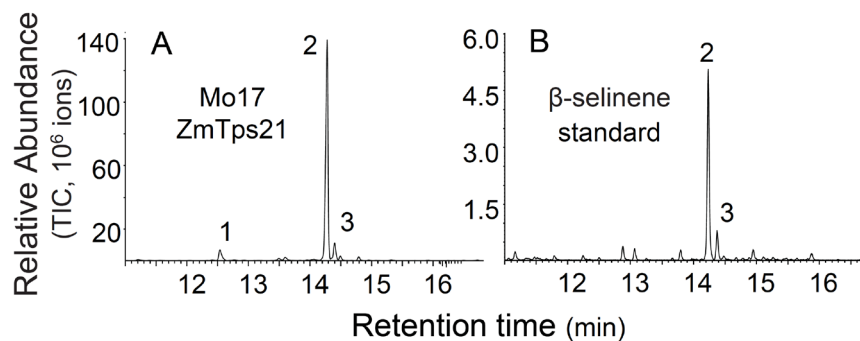


Figure 4. Mo17 *ZmTps21* encodes a functional β -selinene synthase. (A) Mo17 *ZmTps21* was in *Escherichia coli* and the resulting protein extract was incubated with (*E,E*)-farnesyl diphosphate (FPP). Mo17 *ZmTps21* products were collected using solid-phase microextraction and analyzed by GC/MS revealing (2) β -selinene as the dominant product with lower yet detectable levels of (1) β -elemene (germacrene A rearranged product) and (3) α -selinene. (B) Celery fruit essential oil was used as a natural product standard for β -selinene/ α -selinene (9:1).

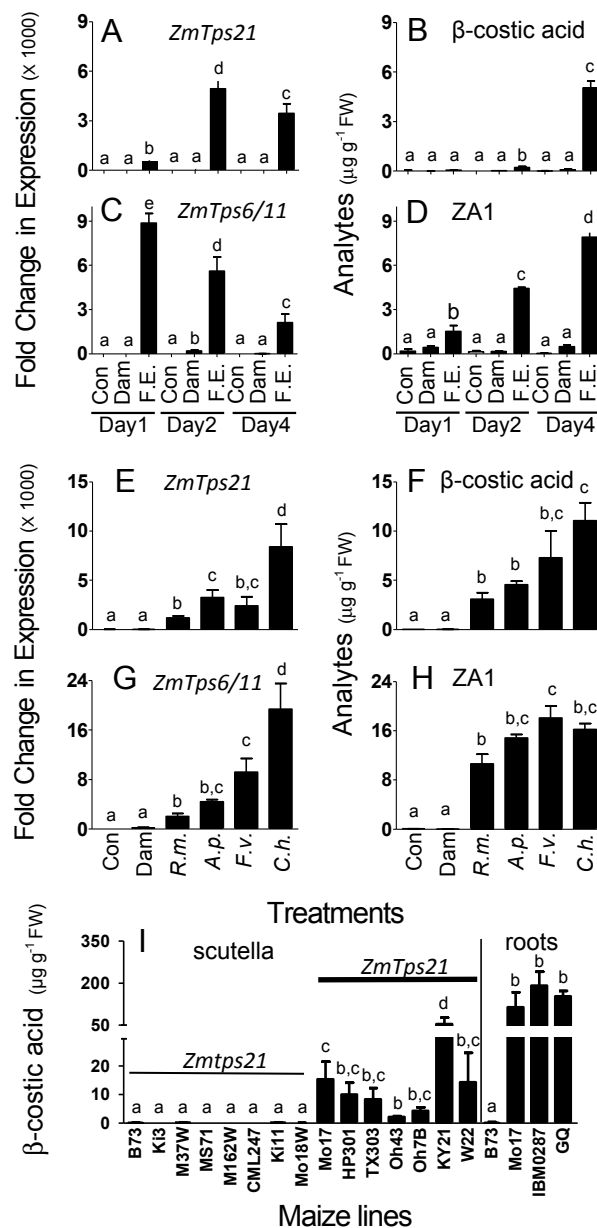


Figure 5. ZmTps21 transcripts are elicited by diverse fungi and precede β-costic acid accumulation detectable in diverse maize lines. Average (n = 4; ±SEM) Mo17 (A) *ZmTps21* (B) β-costic acid (C), *ZmTps6/11* and (D) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations (μg g⁻¹ FW) in intact control stems (Con) or those damaged and treated with either H₂O (Dam), or a heat-killed *Fusarium* elicitor (F.E.) hyphae preparation after 1, 2, or 4 days. Average (n = 4; ±SEM) Mo17 (E) *ZmTps21* (F) β-costic acid (G), *ZmTps6/11* and (H) zealexin A1 as qRT-PCR fold changes of transcripts and corresponding phytoalexin concentrations (μg g⁻¹ FW) in intact control stems (Con) or those damaged and treated with either 100 μl of H₂O (Dam) alone or spore suspensions (1×10⁷ ml⁻¹) of *R. microsporus* (R.m.), *A. parasiticus* nor-1 (A.p.), *F. verticillioides* (F.v.), or *C. heterostrophus* (C.h.) and harvested at 2 and 4 days for transcripts and metabolites, respectively. (I) Average (n = 4, ± SEM) β-costic acid concentrations (μg g⁻¹ FW) in the scutella of 10-d-old maize seedlings from 15 inbred maize lines and mature field collected roots displaying necrosis. Hybrids include sweet corn (var. Golden Queen; GQ) and IBM-RIL0287. Within plots, different letters (a-e) represent significant differences (all ANOVA *P* < 0.05; Tukey test corrections for multiple comparisons: *P* < 0.05).

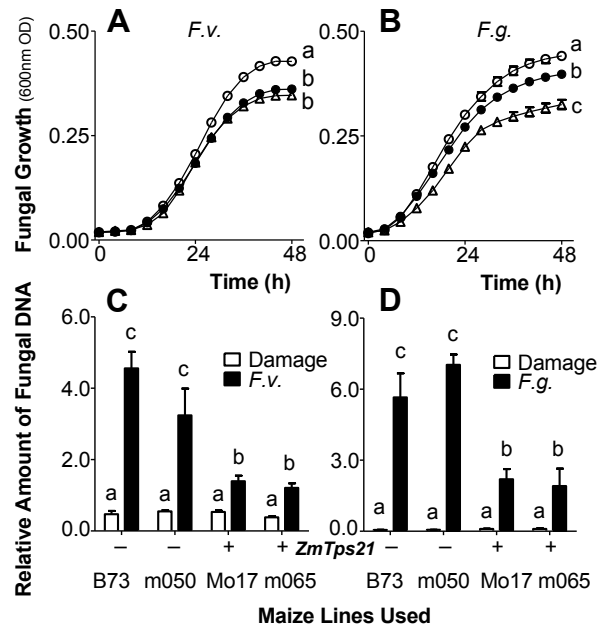


Figure 6. ZmTps21 derived products inhibit *Fusarium* fungi *in vitro* and correspond with improved disease resistance *in vivo*. Average ($n = 8$, \pm SEM) fungal growth estimates (600 nm OD) of (A) *F. verticillioides*, and (B) *F. graminearum*, in liquid media in the presence of β -costic acid at 0 (\circ), 25 (\bullet), and 100 (Δ) $\mu\text{g ml}^{-1}$. Average ($n = 4$, \pm SEM) ratio of fungal DNA / plant DNA levels present in maize roots 7 days after damage and inoculation with 100 μl of either H_2O or 1×10^7 conidia ml^{-1} of (C) *F. verticillioides* and (D) *F. graminearum* in B73, Mo17 and IBM-NILs harboring active (+; m065) and inactive (-; m050) alleles of *ZmTps21*. Within plots, different letters (a–c) represent significant differences (All ANOVA $P < 0.05$; Tukey test corrections for multiple comparisons: $P < 0.05$).

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